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The estrogen receptor (ER) regulates the expression of genes involved in the growth, proliferation and differentiation of skeletal, cardiovascular, neural and reproductive tissues. A basic scheme for the mechanism for ER action has been developed, but precise details on the interactions between ER and the cellular signaling and transcription machinery required for receptor-mediated regulation of specific target genes are still lacking. We have developed an estrogen responsive system in the fruit fly, *Drosophila melanogaster* in order to explore the functional interactions between ER and other cellular proteins. Transgenic flies carrying the human ER alpha and an estrogen responsive green fluorescent protein (GFP) reporter gene were constructed. *In vivo* expression of the GFP reporter gene was observed when larvae were grown on a food source containing steroidal or nonsteroidal estrogens. The induction of the reporter gene by estrogens was blocked upon treatment with tamoxifen, an estrogen antagonist. The polytene chromosomes of *Drosophila* larval salivary glands will be used to identify transcription cofactors and complexes recruited to an estrogen responsive promoter *in vivo*.

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Daryl C. Thackeray 6/9/02

PI - Signature Date

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Introduction

Steroid receptors regulate the expression of many genes involved in human development, metabolism and homeostasis. Ectopic expression of steroid receptors in a genetically amenable organism such as the fruit fly, *Drosophila melanogaster* could provide details on the interactions between ER and the cellular signaling and transcription machinery required for receptor-mediated regulation of specific target genes. There is a significant amount of conservation of cell signaling pathways and the transcriptional apparatus between mammals and fruit flies. Not only does *Drosophila* possess homologues to many mammalian signaling proteins, chromatin remodeling factors, coregulators and basal transcription factors but *Drosophila* also express nuclear receptors. These nuclear receptors include the ecdysone receptor (EcR) that binds the steroid hormone, ecdysone, and *ultraspiracle* (*usp*), the *Drosophila* homologue to the retinoid X receptor. The fact that EcR is used as part of an inducible expression system in mammalian cells and that the glucocorticoid receptor functions in cultured *Drosophila* cells suggests that many insect and mammalian transcription factors are functionally interchangeable. We have developed an estrogen responsive system in the fruit fly, *Drosophila melanogaster* in order to explore the functional interactions between ER and other cellular proteins. The polytene chromosomes of *Drosophila* larval salivary glands will be used to identify transcription cofactors and complexes recruited to an estrogen responsive promoter *in vivo*.

Annual Summary

Aim One: Develop an estrogen responsive fly in which a reporter gene is regulated by estrogens.

Aim Two: Determine how estrogen receptor agonists and antagonists behave in the transgenic fly compared to mammalian cells.

Published paper summarizes our results for the first two aims. Thackray, V., Young, R., Hooper, J., and Nordeen, S. (2000) Estrogen Agonism and Antagonism on the Human Estrogen Receptor in *Drosophila*. Endocrinology, 141(10), 3912-3915

Aim Three: Use polytene chromosomes of *Drosophila* larval salivary glands to directly assess the recruitment of transcription cofactors by ER to an ERE-containing target gene *in vivo*.

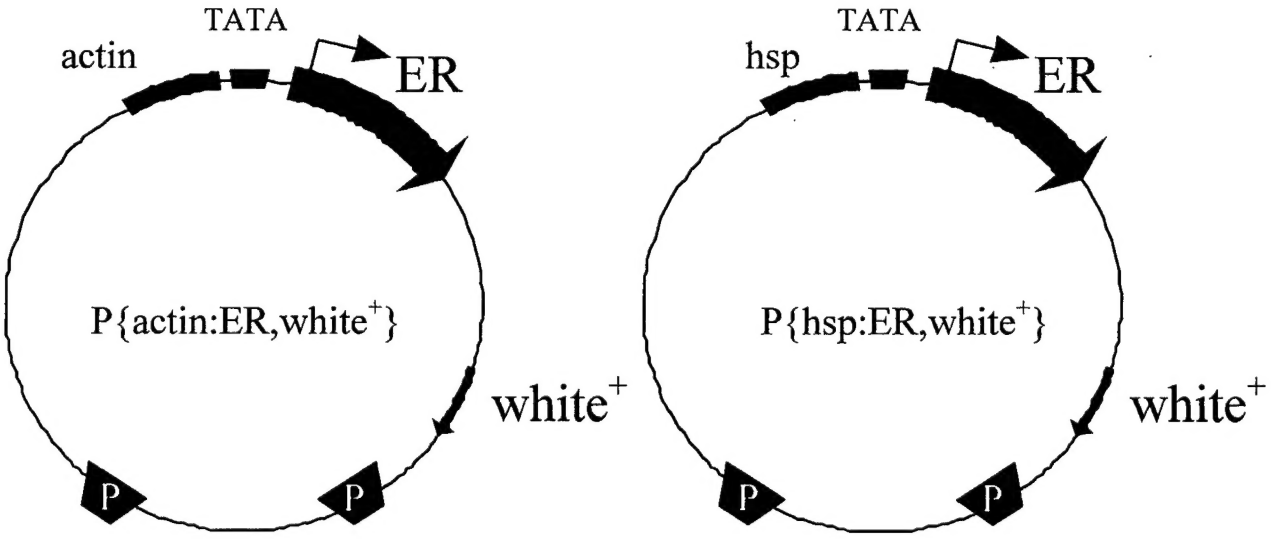

Figure 1. P element transposons for constitutive and heat shock dependent expression of ER.

Last year, I used standard P element-mediated transformation technology to generate the two transgenic fly lines described below. Schematics of the P element vectors are shown in Figure 1. In order to create a fly containing ER in its salivary glands, I constructed two transgenic fly lines by P-element mediated transposition. The first contained a transposon that allows the expression of ER under the control of the actin promoter. This results in constitutive ER expression throughout the fly body. The eye color marker *white+* is linked to the actin:ER to facilitate initial detection of the transgenic flies as well as later mapping and genetic manipulations. Since I did not obtain any transgenic flies carrying the actin:ER transgene, I presume that constitutive expression of ER is toxic to the fly. Therefore, I also constructed a second fly line containing a

transposon that regulates the expression of ER under the control of the heat shock promoter. The eye color marker *white+* is linked to the hsp:ER to facilitate initial detection of the transgenic flies as well as later mapping and genetic manipulations.

Once I obtained the hsp:ER transgenes, I determined whether ER was expressed in the fly after heat shock. Initially, *Drosophila* embryos were tested to see if the expression of ER was heat shock inducible. Male and female flies carrying the hsp:ER transgene were crossed and embryos were collected. The embryos were heat shocked in an eppendorf tube for 1 hour at 37°C. Then the embryos were allowed to recover from the heat shock for 10, 30, 60 or 120 minutes. The embryos were crushed and resuspended in a SDS sample loading buffer containing 10% β-mercaptoethanol. The samples were run out on a 7.5% SDS-Page gel and then analyzed by Western blot analysis using a monoclonal antibody against ER. The highest expression of ER protein occurred in embryos that were allowed to recover for at least 120 minutes.

I then tested whether there was a similar recovery profile in the 3rd instar larvae of the fly. The polytene chromosomes in the salivary glands of 3rd instar larvae will be used to assess the recruitment of transcription cofactors by ER to an ERE-containing target gene *in vivo*. Analysis of ER expression in 3rd instar larvae was problematic compared to fly embryos because it was more difficult to heat shock the larvae and extract the protein. The larvae were put into an eppendorf tube and were heat shocked, fully submerged, for 1 hour at 37°C. Then the larvae were allowed to recover from the heat shock for 10, 30, 60 or 120 minutes. The larvae were crushed and resuspended in a SDS sample loading buffer containing 10% β-mercaptoethanol. The samples were run out on a 7.5% SDS-Page gel and then analyzed by Western blot analysis using a monoclonal antibody against ER. The highest expression of ER protein occurred in larvae that were allowed to recover for at least 120 minutes. I then heat shocked the larvae and allowed them to recover for a longer period of time to see if ER expression would increase even further. The larvae were allowed to recover for 1, 2, 3, 4, 5, 6, 7 or 8 hours after heat shock. The optimal level of ER expression in the 3rd instar larvae occurred 4-6 hours after heat shock treatment. I also tested whether ER was expressed in the salivary glands of 3rd instar larvae. The larvae were heat shocked and allowed to recover for 5 hours. Then the salivary glands were dissected and ER was detected using immunohistochemistry with an ER monoclonal antibody. ER was expressed throughout the salivary gland after heat shock treatment.

Since I had generated several different fly lines carrying the hsp:ER transgene, I determined which lines expressed the highest level of ER. The amount of ER expression was analyzed in the embryo and 3rd instar larvae for lines 1.3, 3.2, 4.3, 5.2 and 7.2. All of the lines expressed ER except for line 5.2 which had a very low level of expression. This low level of expression was probably due to a different chromatin environment surrounding this integrated transgene i.e. the transgene could be near a silenced region of the genome. Lines 1.3 and 3.2 were chosen for further analysis. The transgenes in these lines were mapped to chromosome two and three respectively.

Double transgenic flies containing an hsp:ER transgene and an ERE:GFP reporter gene were generated. Line 1.3 (hsp:ER on chromosome two) was crossed to lines 11.1 or 11.2 (ERE:GFP on chromosome III). Line 3.2 (hsp:ER on chromosome three) was crossed to lines 10.5 or 10.6 (ERE:GFP on chromosome two). The double transgenic flies carrying the hsp:ER and the ERE-

GFP reporter gene were then assessed for estrogen responsiveness. The progeny were raised on media containing estradiol or vehicle control. Third instar larvae were collected and observed using a Zeiss microscope at 200x magnification with a fluorescein isothiocyanate (FITC) filter. I observed induction of the GFP reporter gene in the presence of hormone when the larvae had received a heat shock treatment but not with hormone or heat shock alone.

While I was generating the double transgenic flies, I obtained antibodies for several transcription cofactors that are believed to be involved in estrogen responsiveness. The polytene chromosomes in the salivary glands of 3rd instar larvae will be incubated with these antibodies to assess the recruitment of these cofactors by ER to the ERE:GFP reporter gene. I have collected antibodies from commercial and academic sources that recognize *Drosophila* chromatin remodeling proteins such as SWI/SNF and HMG as well as coactivators such as SRC-1, CBP, p/CAF and general transcription factors such as TBP and TAFs.

My experiments have demonstrated that human steroid receptors can function in *Drosophila melanogaster* and suggest that *Drosophila* genetics could be applied to dissecting the mechanisms of receptor action. I plan to use the polytene chromosomes of *Drosophila* larval salivary glands to visualize the recruitment of factors to an estrogen-responsive promoter *in vivo*. One advantage of a genetic strategy is that it would not be biased by preconceptions about the mechanism of steroid receptor action.

Key Research Accomplishments

- Developed an estrogen responsive system in the fruit fly, *Drosophila melanogaster* in order to use the polytene chromosomes of *Drosophila* larval salivary glands to directly assess the recruitment of transcription factors by ER to an ERE-containing target gene *in vivo*.

- Determined that ER is expressed in a heat shock inducible manner in the hsp:ER transgenic fly in the embryo and 3rd instar larvae.

- Established the heat shock conditions necessary for optimal ER expression in the salivary glands of 3rd instar larve containing the hsp:ER transgene.

- Constructed double transgenic fly lines carrying the P{hsp:ER, white+}and P{ERE:GFP, white+} transgenes. Observed estrogen responsiveness with double transgenic flies.